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Development of an ELISPOT assay for HSV-1 and clinical validation in lung transplant patients

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1 **Development of an EliSPOT assay for HSV-1 and clinical validation in lung transplant**
2 **patients.**

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8

9 **Runing title.** Elispot for HSV-1.

10

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20 **SUMMARY**

21 Cellular immunity plays a relevant role in control of HSV-1 infection/reactivation with a potential
22 impact on clinical-therapeutic management of immunocompromised patients, such as transplant
23 recipients.

24 Herein, we quantitatively evaluated T-cell response directed at HSV-1 by a newly developed IFN- γ
25 EliSPOT assay in 53 patients (including 45 lung transplant recipients and eight subjects in waiting
26 list).

27 Overall, 62.2% of transplant patients and 62.5% of subjects in waiting list evidenced a response to
28 HSV-1 with no significant difference in the level of virus-specific cellular immunity. Response
29 tended to be lower in the first 3 months posttransplantation with progressive recovery of
30 pretransplantation status by the second year and in the presence of HSV-1 DNA positivity in
31 bronchoalveolar lavage. As expected, no response was found in seronegative patients. No
32 significant difference in the level of response according to IgM and IgG status was found.

33 Further studies are required to define the role of HSV-1 specific immune response for the clinical-
34 therapeutic management of lung transplant patients and in other clinical settings and to define cut-
35 off levels discriminating between absence/low and strong response to be related to the risk of viral
36 infection/reactivation.

37

38 **Key words:** herpes simplex virus type 1; cellular immune response; EliSPOT assay; lung
39 transplantation.

40 **Introduction**

41 Herpes simplex virus type-1 (HSV-1) is a highly seroprevalent and ubiquitously distributed dsDNA
42 virus belonging to the *Herpesviridae* family, α -herpesvirinae subfamily. Primary infection usually
43 occurs early in the childhood and is followed by a lifelong latent infection in neurons of central
44 ganglia, from which reactivation may occur. Whereas asymptomatic mucosal shedding is common
45 and HSV-1 has been isolated from the saliva of 1-5% of healthy subjects (Tsakris and Pitiriga
46 2011), reactivation has been reported particularly in immunosuppressed and critical patients in
47 which, beside classical presentation, visceral or disseminated disease can occur, including extensive
48 mucocutaneous involvement, hepatitis, meningoencephalitis, and pneumonitis (Tsakris and
49 Pitiriga 2011; Simmonds-Smit et al. 2006; Costa et al. 2012c; Wilk et al. 2013; Preiser et al. 2003;
50 Bonizzoli et al. 2016). As regards the lower respiratory tract, HSV-1 has been reported in 16 up to
51 32% of the cases (Bruynseels et al. 2003; van den Brink et al. 2004; Daubin et al. 2005; Luyt et al.
52 2007; Linssen et al. 2008) and has been increasingly associated to pulmonary diseases, with poor
53 outcome and high mortality rates (Costa et al. 2012c; Luyt et al. 2007; Linssen et al. 2008; Ong et
54 al. 2004; Engelmann et al. 2007; Gooskens et al. 2007; De Vos et al. 2009; Bouza et al. 2011;
55 Scheithauer et al. 2010). Adaptive immunity plays a pivotal role in uncomplicated recovery from
56 HSV infection, as evidenced by severe complications observed in immunocompromised
57 individuals, although the kinetics and specificity of HSV-specific T-cells during primary infection
58 are poorly unknown (Ouwendijk et al. 2013). After resolution of acute infection, memory T-cells
59 are detected at moderate levels in blood of immunocompetent subjects, with a poly-specific T-cell
60 response directed at distinct HSV-1 tegument and capsid proteins (Jing et al. 2012; Moss et al.
61 2012). Blood HSV-specific T-cells express high levels of cytolytic molecules and secrete IFN- γ
62 upon antigenic recall (Ouwendijk et al. 2013); higher levels of IFN- γ production are associated with
63 polyfunctionality of T-cells and better control of chronic viral infection (Merindol et al. 2012;
64 Harari et al. 2006). Moreover, HSV-1-specific T-cells localize to sites of primary and recurrent
65 infections, as well as latency sites, contributing to control viral latency and reactivation (Ouwendijk
66 et al. 2013; Khanna et al. 2003; Gebhardt et al. 2009; Ariotti et al. 2012). Quantitative evaluation of
67 HSV-1-specific T-cell response in blood compartment and the study of the relation between this and
68 ability of controlling local reactivation in the lung could be relevant for the clinical management of
69 immunocompromised patients at risk of severe pulmonary complications. At moment, no assay for
70 evaluation of cellular immune response to HSV-1 is available, as well as no data on its potential

71 impact on clinical/therapeutic management of infection/reactivation in different categories of
72 patients have been evaluated.

73 In the present study, we quantitatively evaluated T-cell responses directed at HSV-1 by an newly
74 developed IFN- γ EliSPOT assay in a susceptible population such as lung transplant recipients and
75 investigated the role of systemic virus-specific immunity in determining the risk of viral
76 reactivation in the lower respiratory tract.

77

78

79 **Materials and methods**

80 *Subjects and specimens*

81 Cellular immune response to HSV-1 was evaluated in an observational, longitudinal and
82 prospective study by IFN- γ EliSPOT assay on peripheral blood mononuclear cell (PBMC)
83 specimens from all lung transplant recipients admitted to the University Hospital “Città della Salute
84 e della Scienza di Torino”, Turin, Italy (Regione Piemonte Transplant Centre) over a two-year
85 period. The Lung Transplant Centre of the Piemonte region is the first in Italy for activity volume.
86 Overall, 53 patients (M/F, 33/12; mean age \pm standard deviation, 47.8 ± 15.2 years; range, 16-69),
87 including 45 lung transplant recipients in the first two years posttransplantation and eight subjects
88 in waiting list were prospectively evaluated. In this study population, one (in subjects in waiting
89 list) or at least three (in transplant patients) PBMC specimens were collected, accounting for an
90 overall number of 168 samples (160 from transplant recipients, including 81 from 27 patients with
91 three evaluations, 64 from 16 patients with four evaluations, and 15 from three patients with five
92 evaluations; eight specimens from individuals in waiting list). A pre-transplant evaluation of HSV-1
93 cellular immune response was also obtained for all the patients, but three. Pretransplant serological
94 data for HSV-1/2 (IgG and IgM serostatus) were extrapolated from the local Transplant Registry
95 and were available for all patients, in particular five individuals IgG-negative and 48 IgG-positive,
96 with five subjects being IgM-positive. Baseline characteristics of the enrolled patients are reported
97 in Table 1. Moreover, 42 healthy seropositive individuals (IgG-positive, IgM-negative), including
98 38 without recurrent HSV-1 infection and four with at least one episode of HSV-1 infection (herpes
99 labialis) in the previous 12 months, were also studied by a single EliSPOT determination.

100 All subjects provided written informed consent and the study was approved by the institutional
101 review board. According to our lung transplant center’s practice, all patients received prophylaxis
102 for HSV consisting in administration of acyclovir (400 mg twice daily; to be reduced in case of
103 kidney failure or suspended in case of ganciclovir or valganciclovir treatment for CMV). In
104 addition, all patients received a universal, prolonged and combined anti-viral prophylaxis for CMV,
105 irrespective of serological matching donor/recipient, consisting in the administration of ganciclovir
106 or valganciclovir (450 mg twice daily) from day 21 posttransplantation for 3 weeks associated to
107 CMV-Ig (Cytotect Biotest) at days 1, 4, 8, 15, and 30 (1.5 ml/kg body weight) and monthly up to 2
108 years posttransplantation, according to local practice. Long-term immunosuppression was
109 maintained with tacrolimus or cyclosporine A (in patients with cystic fibrosis as underlying

110 disease), mycophenolate mofetil and prednisone (to be tapered or discontinued). Follow-up
111 surveillance bronchoscopies (with bronchoalveolar lavage [BAL] and transbronchial biopsy) were
112 scheduled at 1, 3, 6, 9, 12, 18, and 24 months posttransplantation, for the evaluation of rejection and
113 infections in the lower respiratory tract, as previously described (Costa et al. 2012a; Costa et al.
114 2008; Costa et al. 2011; Costa et al. 2012b). Therefore, virological data for HSV-1 were available
115 on BAL specimens concomitantly collected with samples for EliSPOT assay in all the cases. HSV-1
116 was evaluated on BAL specimens by real-time PCR using a commercially available kit (HSV-1
117 ELITe MGB® kit, ELITechGroup) following automated extraction with the Qiasymphony (Qiagen,
118 Hilden, Germany) instrument. Rapid shell vial isolation with indirect immunofluorescence for
119 HSV-1 was also performed, as previously described (Costa et al. 2007).

120

121 *IFN- γ EliSPOT assay*

122 HSV-1 antigenic stimulus consisted of a freeze-thaw/sonicated viral lysate prepared from expanded
123 long-term cultures of Vero cells (kidney epithelial cells from African green monkey, as previously
124 described (Terlizzi et al. 2009), infected with the Human herpesvirus 1 ATCC® VR-260™
125 [American Type Culture Collection, Manassas, VA, USA]). Aliquots of viral preparation were
126 stored at -80°C until use. For virus titration, 96-well plates at 60-80% confluence of Vero cells were
127 inoculated with 100 μ l of 10-fold diluted virus for TCID₅₀ assay, obtaining an end-point titer of
128 3.16×10^8 TCID₅₀/ml. Sonication included thawing of the virus in ice and 3 cycles at 20% intensity
129 for 30 seconds using the Sonopuls Ultraschall-Homogenisatoren instrument (Bandelin electronic
130 GmbH, Berlin, Germany). Subsequently, the virus underwent a through UV irradiation for
131 inactivation, with two cycles per transilluminator set at 1.2 J/cm². UV inactivation was carried out
132 also on the RPMI 1640-medium (Sigma-Aldrich, St. Louis, MO, USA), used for the EliSPOT assay
133 (see below). In order to ascertain the effective inactivation of the virus, a rapid shell vial culture
134 assay followed by indirect immunofluorescence was performed, as previously described (Costa et
135 al. 2007), and resulted negative (Figure 1). For antigenic stimulus, serial dilutions from 10⁶ up to
136 10³ of the inactivated virus, starting at 3.16×10^8 TCID₅₀/ml were used. Dose response curves were
137 performed with the lysate preparation to determine the amount of antigenic stimulus to use in the
138 IFN- γ EliSPOT assay: in particular, on PBMCs obtained from four healthy controls and two lung
139 transplant recipients.

140 Whole blood was collected directly into CPT Vacutainer tubes (BD, Franklin Lakes, NJ, USA) and
141 PBMCs were separated by density gradient sedimentation according to manufacturer instructions,
142 with minor modifications. Briefly, blood samples were centrifuged at 1800 g for 20 min at room
143 temperature. The resulting mononuclear cell fraction was washed twice with phosphate buffered
144 saline (PBS 1x, pH 7.4). Resulting PBMCs were cryopreserved in fetal calf serum (PAA
145 Laboratories GmbH, Pasching, Austria) with 10% dimethyl sulfoxide, placed into Nalgene
146 Cryovials (Nalge Nunc, Rochester, NY, USA) at -80°C for ≥ 24 h prior to transfer to liquid nitrogen
147 for long-term storage.

148 The IFN- γ EliSPOT assay was performed as described elsewhere (Costa et al. 2012b). Briefly,
149 PBMCs were thawed in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10%
150 fetal calf serum and 1% l-glutamine, washed twice and rested for at least 4 h in complete RPMI-
151 1640 at 37°C, 5% CO₂, before assay. Subsequently, cell viability and count were assessed by trypan
152 blue staining in Burker's chamber to a final concentration of 2×10^6 cells/ml. Peripheral blood
153 mononuclear cells were plated at 2×10^5 cells/well onto a 96-well microplate pre-coated with anti-
154 human IFN- γ monoclonal antibody (EliSPOT Interferon- γ Basis Kit; AID, Strassberg, Germany)
155 and incubated with viral preparations, as described above. For negative and positive controls, cells
156 incubated with supplemented RPMI -1640 medium alone and with 1 μ g/ml phytohemagglutinin
157 mitogen (supplied by ELITechGroup, Milan, Italy) were used, respectively. Following a 18-20 h
158 incubation at 37°C, 5% CO₂, the microplates were washed 8-times with washing buffer and
159 incubated with biotinylated anti-human IFN- γ mAb at 1 μ g/ml in VP buffer at room temperature in
160 a wet chamber, in the dark, for 2 h. Subsequently, the microplates were washed 8-times with
161 washing buffer and incubated with streptavidin-horseradish peroxidase solution diluted 1:1000 in
162 blocking buffer. Following another washing step, as before, substrate solution
163 (tetramethylbenzidine) was added for colour development at room temperature in the dark for 12-15
164 min. The chromogenic reaction was stopped by extensive washing with tap water and microplates
165 were allowed to completely dry before analysis. Results were analyzed using a computer-assisted
166 system (AID EliSPOT Reader System, AID). Data were expressed as spot-forming units (SFU)/ $2 \times$
167 10^5 cells, with each spot representing a single cell that produces IFN- γ , calculated by subtracting the
168 mean of SFU obtained in unstimulated negative control from the mean SFU obtained in the antigen-
169 stimulated wells.

170 *Statistical analysis*

171 For descriptive statistics, data were expressed as raw number and percentage. For statistical
172 analysis, chi square, t test, and analysis of variance (ANOVA, followed by Bonferroni post-test)
173 were applied, as appropriate. A p value <0.05 was considered significant. Statistical analysis was
174 performed using GraphPad Prism version 5 (GraphPad Software, San Diego, USA).

175

176

177 **Results**

178 *Validation of the EliSPOT assay*

179 Based on dose-response curves on preliminary EliSPOT assays performed in triplicate on PBMCs
180 from four IgG positive healthy controls and two IgG-positive lung transplant patients, serial HSV-1
181 lysate dilutions at 3.16×10^4 and 3.16×10^3 TCID₅₀/mL were associated to more robust and
182 reproducible responses, even though not at statistical level ($p = \text{n.s.}$)(Figure 2). Therefore, these
183 dilutions were used as antigenic stimuli for subsequent HSV1-EliSPOT evaluations on specimens
184 from study population. The results evidenced that 3.16×10^3 TCID₅₀/mL HSV-1 stimulus was
185 associated to higher responses in comparison to 3.16×10^4 TCID₅₀/mL (mean SFU/2 $\times 10^5$ PBMCs
186 \pm SD; 3.765 ± 5.516 versus 2.662 ± 4.531 , $p=0.048$), when considering samples from
187 posttransplantation setting (peak value of response for each patient)(Figure 3).

188

189 *Clinical evaluation*

190 Overall, 28/45 (62.2%) transplant patients and 5/8 (62.5%) patients in waiting list evidenced a
191 positive response to HSV-1 lysate, with level of response ranging from 1 to 211 and from 8 to 53
192 SFU/2 $\times 10^5$ PBMCs, respectively. No significant difference of response was found between
193 samples from pre- and post-transplant patients, considering both all specimens (mean SFU/2 $\times 10^5$
194 PBMCs \pm SD, 8.9 ± 15.9 versus 7.2 ± 25.6 ; $p = 0.870$) and specimens with peak value from each
195 patient (10.4 ± 18.1 versus 5.15 ± 25.6 ; $p = 0.120$). Subsequently, as the main risk of HSV-1
196 infection is in the first period posttransplantation (up to 3 months, particularly the first 30 days), we
197 compared the degree of specific cellular response in specimens collected in the first 3 months
198 (overall, 10 specimens from as many patients) versus those collected at > 3 months and found no
199 significant differences, although mean values of SFU/2 $\times 10^5$ PBMCs tended to be lower in the early
200 period in comparison to >3 months (mean \pm SD, 2.7 ± 5.5 versus 7.4 ± 27.3 ; $p = 0.638$).

201 Among healthy seropositive individuals, 39/42 (83.3%) evidenced a positive response to HSV-1
202 lysate (37 with no episode of recent HSV-1 infection in the previous 12 months, two with two
203 episodes of herpes labialis), with level of response ranging from 8 to 36 SFU/2 $\times 10^5$ PBMCs; in
204 three subjects no response was found (one with no episode of HSV-1 infection and two with an
205 episode of herpes labialis in the previous 12 months). No significant difference of response was
206 found between individuals with and without HSV-1 infection.

207 Considering serostatus, as expected, no response was found in seronegative patients, whereas a
208 mean level of 11.2 SFU/2X10⁵ PBMCs (range, 0-211; median, 3) was found in seropositive
209 patients, with no significant difference in IgM-positive versus IgM-negative patients (mean SFU/2 x
210 10⁵ PBMCs \pm SD, 3.5 \pm 4.7 versus 11.6 \pm 33.6; p = 0.585). In Table 2, HSV-1 responses to different
211 antigenic concentrations according to IgG serostatus are reported.

212 In order to investigate the kinetics of HSV-1-specific T-cell immunity after lung transplantation,
213 EliSPOT data were evaluated at different time points, including prior to transplantation, at 1 month
214 and at 6-month intervals posttransplantation. The pattern of HSV-1 specific cellular immune
215 response evidenced a decrease in the first months posttransplantation in comparison to
216 pretransplantation levels; this was seen with both 3.16 x 10³ and 3.16 x 10⁴ TCID₅₀/mL antigenic
217 stimuli (mean SFU/2 x 10⁵ PBMC \pm SD, 2.889 \pm 5.061 versus 9.5 \pm 16.10, and 1.0 \pm 2.0 versus 8.2
218 \pm 15.30, respectively), with progressive recovery of pretransplantation levels at the end of the
219 second year posttransplantation (5.444 \pm 8.819 versus 4.222 \pm 6.685, for 3.16 x 10³ and 3.16 x 10⁴
220 TCID₅₀/mL HSV-1 stimuli, respectively)(Figure 4, A and B). This kinetics was observed also when
221 excluding patients with HSV-1 DNA positivity on BAL specimens in concomitance with the
222 EliSPOT determinations (n=7)(Figure 4, C and D).

223 Seven lung transplant recipients (15.6%) exhibited at least one episode of HSV-1 lower respiratory
224 tract infection (as determined by molecular detection of HSV-1 DNA on BAL specimens [Costa et
225 al. 2012c]), concomitant to the available EliSPOT assays. All cases of HSV-1 infection occurred in
226 IgG-positive recipients, likely due to viral reactivation. In these patients, the level of HSV-1 cellular
227 immunity tended to be lower in comparison to patients with no HSV-1 DNA positivity, even though
228 not reaching statistical significance (mean EliSPOT values: 1.143 \pm 0.5533 versus 3.967 \pm 0.7295;
229 p=0.1986).

230 In order to assess the impact of pulmonary events of HSV-1 replication on subsequent virus-specific
231 immunity induction, BAL determinations performed in a 6-month period prior to the available
232 EliSPOT assays were retrospectively investigated. Six patients exhibited a history of at least one
233 episode of pulmonary HSV-1 replication in this period; in these patients, HSV-1 EliSPOT response
234 tended to be higher in comparison to patients with no evidence of lower respiratory tract infection in
235 the same interval (mean SFU/2 x 10⁵ PBMC, 8.167 \pm 3.229 versus 3.568 \pm 0.7953, p=0.0656, using
236 3.16x10³ TCID₅₀/mL as antigenic stimulus). Moreover, no relation was found between HSV-1
237 EliSPOT responses and the occurrence of HSV-1 positivity in the subsequent 6-month period.

238 Discussion

239 In this study, cellular immune response to HSV-1 was evaluated by a newly developed IFN- γ
240 EliSPOT assay. Whereas Posavad and colleagues described an EliSPOT assay for HSV-2 to be used
241 in vaccine development (Posavad et al. 2011), an assay specifically designed for HSV-1 has not
242 been reported in literature and its availability could be useful for defining the role of cellular
243 immunity in the development and outcome of HSV-1 infection/reactivation, as well as in its clinical
244 and therapeutic management. Immunocompromised patients, such as transplant recipients, present
245 more frequent and severe clinical manifestations of HSV-1 infection, as well as decreased responses
246 to anti-viral treatment (Wilk et al. 2013). In most of the cases, symptomatic HSV-1 disease in adult
247 transplant recipients results from viral reactivation, particularly in the first month following
248 transplantation (Fishman 2007). Among other clinical manifestations, including disseminated
249 mucocutaneous disease, esophagitis and hepatitis, pneumonitis is described in all solid organ
250 transplant patients, but most commonly in lung and heart-lung transplant patients (Smyth et al.
251 1990). The kinetics and specificity of HSV-1 T-cell immune response during primary infection are
252 poorly known in humans. Following resolution of acute episode, specific memory T-cells are found
253 at moderate levels of 0.1-1% in immunocompetent individuals (Ouwendijk et al. 2013; Jing et al.
254 2012; Moss et al. 2012). In healthy individuals, a complex and poly-specific CD4⁺ and CD8⁺
255 response towards more than 70 different proteins has been identified, including proteins abundantly
256 present in the virion (e.g. viral envelope, tegument, capsid) and regulatory proteins (Jing et al. 2012;
257 Merindol et al. 2012; Harari et al. 2006; Jing et al. 2013). HSV-1 specific T-cells localize to sites of
258 primary, recurrent and chronic latent infections from which reactivation may occur in favoring
259 conditions, such as immunosuppression. Several studies have demonstrated that the outcome of
260 these infections depends on the efficacy of specific cellular immune response (Remakus and Sigal
261 2013; Sant and McMichael 2012; Calarota et al. 2015) and that the development of quantitative,
262 sensitive and reproducible assays for evaluation and monitoring of virus-specific T-cell response is
263 fundamental to investigate kinetics of HSV-1-specific immunity and in the clinical-therapeutic
264 management of immunocompromised patients.

265 Among methods developed for evaluating virus-specific T-cell response, the EliSPOT assay allows
266 for measurement of quantity and functionality of specific T-cells and can be used to define the
267 whole repertoire of cellular responses without MHC-restriction. EliSPOT assay detects production
268 of IFN- γ by PBMCs following stimulation with specific antigens and enumerates responsive cells
269 using anti-IFN- γ monoclonal antibodies coated onto 96-well plates and a second enzyme-

270 conjugated monoclonal antibody; spots are counted using automated EliSPOT readers with each
271 spot representing a single specific cell (Calarota et al. 2015). The most common antigenic stimuli
272 used for EliSPOT assay are pools of overlapping peptides, peptide libraries spanning entire proteins
273 or viral lysates. Given the antigenic complexity of herpesviruses which contain multiple potential
274 protein targets recognized by CD4+ cells and the dose-response curves obtained on preliminary
275 EliSPOT assays, in this study a viral lysate preparation at 3.16×10^4 and 3.16×10^3 TCID₅₀/mL
276 dilutions was used.

277 By using these two dilutions of inactivated virus, we found that 3.16×10^3 TCID₅₀/mL HSV-1
278 stimulus was significantly associated to higher level of response in comparison to 3.16×10^4
279 TCID₅₀/mL. This difference was evidenced in almost all cases with very few exceptions and
280 considering those collected in both the pre- and post-transplant settings; it could be hypothesized
281 that this is due to the degree of saturation of binding sites.

282 As regards HSV-1 specific cellular immune response in study population, there was no significant
283 difference in its level between the pre- and post- transplant period. As the higher risk (Fishman
284 2007) of HSV-1 reactivation is in the very first months (particularly up to 30 days), we evaluated
285 whether this could be attributable, at least partly, to a lower degree of virus-specific cellular
286 immune control. Although the difference was not significant, a tendency to lower levels of response
287 in the first period was found; of course, it should be taken into account the small number of
288 specimens that could have limited the statistical power of these data and the need for increasing the
289 study group. Moreover, it has to be underlined that we consider cumulative data from all the
290 specimens available for a certain period of time posttransplantation, giving the difference in number of
291 samples available at different time points.

292 Knowledge of HSV-1 serostatus, as well as of cell-mediated immune response, may be of great
293 concern to stratify patients at major risk for primary HSV-1 acquisition – either from the allograft or
294 from natural sources – after transplantation, which may be more clinically severe and prolonged due
295 to lack of immunologic memory (Wilck and Zuckerman 2013; Nichols et al. 2003). As expected, no
296 response was found in seronegative patients; on the other side, when considering seropositive
297 patients, no significant difference in the degree of virus-specific response was found between IgG-
298 and IgM-positive individuals, although values tended to be higher in patients with a serological
299 status suggesting previous infection. This observation supports the hypothesis that a higher level of
300 response is achieved following immunological boosting of memory T-cell, as already reported for

301 cytomegalovirus (Costa et al. 2014a; Rittà et al. 2015; Abate et al. 2010), Epstein-Barr virus (Rittà
302 et al. 2015) and polyomavirus BK (Costa et al. 2014b).

303 Given the occurrence of HSV-1 infection/reactivation in the lower respiratory tract and the potential
304 impact in the presence of impaired immune responses, as reported for other herpesviruses (Costa et
305 al. 2007), a study population of lung transplant recipients was chosen for clinical validation of the
306 developed HSV-1 EliSPOT assay and evaluation of kinetics of specific cellular immune response.
307 As expected, a decrease (although not significant) in the level of response in the first months
308 posttransplantation was found in comparison to pretransplantation levels, with progressive recovery
309 of these levels along a period ranging from 3 months to 2 years posttransplantation.

310 As regards HSV-1 infection in the lower respiratory tract, as evidenced by positivity to HSV-1
311 DNA on BAL specimens, all the cases occurred in IgG-positive patients, thus representing viral
312 reactivation. In terms of impact of the level of HSV-1 specific cellular immune response on viral
313 reactivation, although not statistically significant, a tendency to lower levels in the seven patients
314 with at least one episode of infection was observed, with values even lower in the presence of
315 repeated episodes. Moreover, as these data referred to concomitant evaluation of HSV-1 DNA on
316 BAL and EliSPOT assay, we also assessed the impact of pulmonary HSV-1 infection on subsequent
317 level of virus-specific cellular immune response by retrospectively investigating BAL
318 determinations in a 6-month period prior to the available EliSPOT assay. Interestingly, in patients
319 with at least one episode of pulmonary HSV-1 infection in comparison to those with no infection,
320 the degree of cellular immune response tended to be higher, thus supporting the boosting effect of
321 viral replication on the development of HSV1-specific immunity.

322 In conclusion, we have evaluated T-cell responses directed at a HSV-1 in lung transplant patients by
323 a newly developed, specific and quantitative IFN- γ EliSPOT assay and investigated the
324 immunological status and kinetics. The availability of this assay could allow for a patient's tailored
325 clinical-therapeutic management in terms of modulation of immunosuppressive therapy and use of
326 antiviral agents in the presence of HSV-1 infection/reactivation in relation to the occurrence and
327 level of virus-specific response. Further studies on larger and different populations of
328 immunocompromised and immunocompetent patients are required to define the potential of
329 quantitative evaluation of HSV-1 specific cellular immune response in different clinical settings and
330 to define cut-off levels discriminating between absence/low and strong response to be related to the
331 risk of viral infection/reactivation.

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Table 1. Demographic and clinical features of study population. BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; CSA, cyclosporin A; MMF, mycophenolate mofetil; MPA, mycophenolic acid; TAC, tacrolimus; AZA, azathioprine; EVR, everolimus. Details on antiviral prophylaxis are reported in the text.

Features	
Patients, total n	95
Male/female, n	56/39
Mean age (range), years	47.2 (16-69)
Healthy seropositive individuals, n	42
Mean age (range), years	37.5 (21-49)
N. of EliSPOT determinations per patient	1
Pre-transplant patients, n	8
Mean age (range), years	47 (22-65)
N. of EliSPOT determinations per patient	1
Post-transplant patients, n	45
Mean age (range), years	47.0 (16-69)
N. of EliSPOT determinations per patient (mean, range)	3.6 (3-5)
Time of EliSPOT determinations post-transplantation (months – mean, range)	21 (1-94)
Type of lung transplant	
Monolateral	9
Bilateral	36
Underlying disease	
Cystic fibrosis	37 (50.7%)
COPD/emphysema	22 (26.0%)
Idiopathic pulmonary fibrosis	6 (8.2%)
Bronchiectasis	7 (9.6%)
Extrinsic allergic alveolitis	1 (1.4%)
Antiviral prophylaxis (in all transplant patients)	
HSV	Acyclovir
CMV	Ganciclovir or valganciclovir + CMV-Ig
Immunosuppressive regimens	
CSA + MMF	25
CSA + MPA	1
TAC + MMF	15
TAC + MPA	2
TAC + AZA	1
TAC + EVR	1
HSV-1/2 serology at baseline	
IgM+	5
IgM-	48
IgG+	48
IgG-	5

469 Table 2. HSV-1 EliSPOT responses to different antigenic concentration according to IgG serostatus
 470 (mean \pm standard deviation, spot forming units [SFU]/2x10⁵ peripheral blood mononuclear cells
 471 [PBMCs]).

	HSV IgG+ (n = 48)	HSV IgG- (n = 5)	p
3.16x10 ³ TCID ₅₀ /mL	5.447 \pm 1.543	0.2 \pm 0.2	0.24
3.16x10 ⁴ TCID ₅₀ /mL	3.929 \pm 1.335	0.2 \pm 0.2	0.32

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474 Figure 1. Rapid shell vial culture assay with indirect immunofluorescence using Vero cells infected
475 with (A) human Herpesvirus 1 ATCC® VR-260TM, (B) UV-inactivated HSV-1 preparation
476 (dilution 3.16×10^8 TCID₅₀/mL), and (C) UV-treated RPMI-1640 complete medium alone at 24 h
477 post-infection (Fluorescein isothiocyanate; counterstaining with Evans blue 1:10000).
478 Magnification, 25X.

479

480 Figure 2. EliSPOT assay on peripheral blood mononuclear cells from a HSV-1 IgG-positive lung
481 transplant patient stimulated with serial dilution of UV-inactivated HSV-1 preparation: (A) $3.16 \times$
482 10^4 TCID₅₀/mL, (B) 3.16×10^3 TCID₅₀/mL, (C) RPMI-1640 complete medium alone, and (D)
483 phytohemagglutinin mitogen (PHA) 1 µg/mL. Results are reported as spot forming unit
484 (SFU)/ 2×10^5 cells.

485

486 Figure 3. HSV-1 EliSPOT responses according to concentrations of HSV-1 antigenic stimulus in
487 samples from posttransplantation patients (peak value of response for each patient).

488

489 Figure 4. Kinetics of HSV-1 EliSPOT responses according to concentrations of HSV-1 antigenic
490 stimulus considering all specimens from transplant patients (A, 3.16×10^3 TCID₅₀/mL; B, $3.16 \times$
491 10^4 TCID₅₀/mL) and excluding specimens from patients with concomitant HSV-1 positivity on
492 bronchoalveolar lavage (BAL) (C, 3.16×10^3 ; D, 3.16×10^4 TCID₅₀/mL). Determinations are
493 grouped as follows: at pre-transplant (n = 42), up to 1month (n = 7), at 1-6 months (n = 13), at 6-12
494 months (n = 79), at 12-18 months (n = 53), and at 18-24 months (n = 8) post-lung transplantation
495 (LT).